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Titanium-doped cerium oxide nanoparticles protect cells from hydrogen peroxide-induced apoptosis

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Abstract

To develop new nanoparticle materials possessing anti-oxidative capacity with improved physical characteristics, we have studied titanium-doped cerium oxide (CeTiO₂) nanoparticles. CeTiO₂ nanoparticles had a mode diameter of 15–20 nm. These nanoparticles demonstrated catalase activity, and did not promote the activation of hemolytic or cytolytic pathways in living cells. Using surface plasmon resonance enhanced microscopy, we find that these nanoparticles associate with cells. Transmission electron microscopy studies demonstrated that these nanoparticles accumulate within the vacuolar compartment of cells. Importantly, CeTiO₂ nanoparticles decrease hydrogen peroxide-mediated apoptosis of cells as judged by the reduced cleavage of a caspase 3-sensitive label. CeTiO₂ nanoparticles may contribute to deflecting tissue damage in a broad spectrum of oxidant-mediated diseases, such as macular degeneration and Alzheimer's disease.

Keywords

Catalytic nanoparticles; reactive oxygen metabolites; cell toxicity; apoptosis

Introduction

In contrast to their bulk forms, several nanoparticles have the extraordinary ability to carry out chemical transformations traditionally associated with biological enzymes. Included among the enzymologic activities associated with nanoparticles are: peroxidase, oxidase, catalase, and superoxide dismutase (Gao et al., 2007; Korsvik et al., 2007). For example, superoxide dismutase activity has been ascribed to CeO₂ nanoparticles. Moreover, these nanoparticles have been reported to possess anti-oxidative properties within living cells and animals (Chen et al., 2006; Das et al., 2007; Tarnuzzer et al., 2005; Schubert et al., 2006; Niu et al., 2007; Korsvik et al., 2007; Colon et al., 2009; Elswaifi et al., 2009; Singh et al., 2007). Thus, catalytic nanoparticles may have a role in deflecting oxidant-mediated cell damage in human diseases.

Although catalytic CeO₂ nanoparticles have certain advantages as an experimental therapeutic in replacing or augmenting endogenous anti-oxidative enzyme activities, they

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also have limitations. For example, as CeO₂ nanoparticles are crystalline polyhedra (e.g., hexahedral, octahedral, and truncated octahedral), their sharp edges are thought to scratch surfaces during polishing (Feng et al., 2006). Several studies have shown that nanoparticle toxicity may be traced to nanoparticle shape, with spherical nanoparticles being much less toxic to cells than plate-shaped nanoparticles (George et al., 2012; Pal et al., 2007). Thus, nanoparticles bearing flat surfaces may damage both inorganic and biological structures. Furthermore, planar surfaces also contribute to nanoparticle aggregation (Buettner et al., 2010; Keller et al., 2010). As the superoxide dismutase activity of CeO₂ nanoparticles is greater than that of the biological superoxide dismutase enzyme (Korsvik et al., 2007), they may reduce superoxide levels below the concentrations required for normal tissue functions. For example, these nanoparticles may interfere with the low levels of superoxide that serve as crucial signaling molecules in cell migration and proliferation (D'Autréaux and Toledano, 2007; McCubrey et al., 2006). Hence, it is of interest to test nanoparticles that exhibit physical and chemical properties better suited for a biological environment.

We have recently reported that Ti-doped CeO₂ nanoparticles exhibit superoxide dismutase activity that is reduced in comparison to non-doped nanoparticles (Zhu et al., 2012). We now report that CeTiO₂ nanoparticles possess catalase activity and that this enzymatic activity can reduce hydrogen peroxide-induced apoptosis. The physicochemical and biological properties of these nanoparticles make them an attractive agent for pre-clinical studies of disease models of oxidant stress.

Materials and Methods

Materials

Cell culture media, Hank's balanced salt solution (HBSS), and Calcein-AM were purchased from Invitrogen (Carlsbad, CA). The CaspGLOW red active caspase-3 staining kit was obtained from BioVision (Mountain View, CA). Cover-glass bottom dishes were purchased from MatTek Corporation (Ashland, MA).

Nanoparticles

The compositions of nanoparticles used in these studies were: CeO₂ (lot number 6SB232A2), Ce_{0.95}Ti_{0.05}O₂ (lot number 6SB254A), Ce_{0.90}Ti_{0.10}O₂ (lot number 6SB257B), Ce_{0.85}Ti_{0.15}O₂ (lot number 6SB257D) and Ce_{0.875}Ti_{0.125}O₂ (lot number 1SP014A). These nanoparticles were purchased from Nanocerox, Inc. (Ann Arbor, MI). Tungsten oxide nanoparticles (size ~50 nm) were purchased from Sigma Chem. Co. (St. Louis, MO).

Nanoparticle suspensions

Nanoparticles were suspended in HBSS, and maintained at 4 °C. All suspensions were vortexed before use.

Cell culture

Tumor cells were maintained on plastic tissue culture flasks in Dulbecco's modified Eagle medium (DMEM, Invitrogen) containing 10% heat-inactivated FBS (Invitrogen) and 1% antibiotic/antimycotic (Invitrogen), as previously described (Clark et al., 2010, 2011). Cells

were detached using a trypsin/EDTA solution (Invitrogen), transferred to fresh media, and plated onto glass cover slips 24 hr prior to use. For cytolysis assays, cells were plated into 96 well cell culture plates (Costar 3595, Corning Inc., Corning, NY) at 10^4 cells per well in DMEM.

Hemolysis assay

Peripheral blood was collected from healthy human donors in compliance with the guidelines of the University of Michigan Institutional Review Board for Human Subject Research. Red blood cells were isolated using Ficoll-Histopaque (Sigma) density gradient centrifugation, then re-suspended and washed in HBSS by centrifugation. Cells were treated with nanoparticles for 3 hr at 37° C. Cells were centrifuged, and supernatants transferred to a 96 well plate. Absorbance at 540 nm was read on a FlexStation plate reader (Molecular Devices, Sunnyvale, CA). A sample treated with 1% Triton-X 100 (Sigma) in HBSS was used as a 100% hemolysis standard.

Cytolysis assay

Cells were labeled with Calcein-AM (Invitrogen) for 30 min at 37° C, and washed thoroughly with HBSS. Triplicate wells were treated with CeTiO₂ nanoparticles at the concentrations indicated below or with 1% Triton-X 100 in HBSS to serve as a reference sample for 100% release. The plate was then incubated for 3 hr at 37° C. After incubation, supernatants were centrifuged to remove particulates. Supernatants were transferred to a fresh 96 well plate then read on a FlexStation II (Molecular Devices) using an excitation of 494 nm and emission of 517 nm. Fluorescence intensities were averaged over triplicate wells.

Catalase Assay

Catalase catalyzes the conversion of hydrogen peroxide to water and molecular oxygen. The Amplex Red catalase assay kit was employed to detect catalase activity (Invitrogen). Experiments were performed as described by the manufacturer. Briefly, a solution of 2 mg/ml nanoparticles and 0.02 μ M H₂O₂ in PBS was incubated at room temperature for 30 min. with shaking. After removal of nanoparticles by centrifugation, 100 μ l of each control or test solution was added to one well of a multi-well plate. To this solution, an equal volume of PBS containing 50 μ M Amplex Red and 0.1 U/ml horseradish peroxidase was added. After 30 min. the fluorescence was measured using 530 nm excitation and 590 nm emission. Catalase activity was demonstrated by the disappearance of hydrogen peroxide, as judged by the loss of Amplex Red fluorescence.

Apoptosis

Cells were seeded into cover-glass bottom dishes and allowed to grow for 24-48 hr. CeO₂ or CeTiO₂ nanoparticles were added at the concentrations indicated below and then incubated at 37° C. After 24 hr, the plates were washed with HBSS, then incubated for 3 hr at 37° C with either HBSS or HBSS containing 200 μ M H₂O₂. Samples were stained for activated caspase-3 using the CaspGLOW™ red active caspase-3 staining kit (BioVision) as directed by the manufacturer. Plates were washed and then imaged using a Nikon microscope with a

40× objective (see below). Caspase-3 red labeling was observed using a Nikon 96321 filter set comprised of a 570 nm dichroic mirror, a 530-560 nm excitation filter, and a 590-650 nm emission filter.

Bright field microscopy

Cells were seeded onto cover slips and allowed to grow for 24 hr. Cover slips were treated with nanoparticles or buffer alone for an additional 24 hr. Cells were washed with HBSS, and then mounted live for microscopy. Cells were observed using a Nikon microscope equipped with an Andor iXon camera (Andor Techol., Belfast, Northern Ireland) attached to the bottom port of a Nikon TE2000-U inverted microscope that incorporated a 100W mercury lamp for excitation.

Surface plasmon resonance enhanced dark-field microscopy

A CytoViva, Inc. (Auburn, AL) darkfield condenser and illumination source were attached to a Zeiss carrier for transmitted light illumination. A Zeiss Axiovert microscope equipped with a 100× darkfield objective (Olympus) was employed. Surface plasmon resonance enhanced microscopy produces a very high contrast image of high optical resolution (90 nm). To preserve resolution, a 1.9× Optem camera coupler (Qioptiq Imaging Solutions, Fairport, NY) was used to direct the image to a large format (4096 × 4096) Alta-U16M camera (Apogee Imaging Systems, Roseville, CA). The camera was water-cooled to reduce the dark noise and to minimize physical vibrations due to cooling. Images were processed using Metamorph software (Molecular Devices).

Electron microscopy

Nanoparticles were studied as whole mounts on carbon-coated copper grids. For cell studies, untreated control cells or nanoparticle-treated cells were fixed in 1% glutaraldehyde in phosphate-buffered saline for 1 hr. Samples were then post-fixed with 1% osmium tetroxide (Polysciences, Warrington, PA) in cacodylate buffer for 1 hr. Cells were dehydrated in an increasing graded series of ethanol in water. Samples were washed three times in 100% ethanol then treated with an increasing series of Spurr's resin and ethanol. After three washes with 100% resin, the resin was polymerized overnight at 60° C. Thin sections were cut then counterstained with lead citrate and uranyl acetate. Transmission electron micrographs were collected with a Philips CM-100 TEM.

Results

Nanoparticles

TEM studies were performed to assess nanoparticle properties. Fig. 1 shows a TEM image of CeTiO₂ nanoparticles. These nanoparticles are spherical in shape and range in size from ~5 nm to ~115 nm. Fig. 2 shows a plot of the size distribution of the nanoparticles; as shown by this graph, the mode of the distribution was 15-20 nm.

Enzymatic Activities

We have recently reported that CeTiO₂ nanoparticles possess catalytic activities mimicking those of the enzymes superoxide dismutase and oxidase (Zhu et al., 2012). To determine whether CeTiO₂ nanoparticles possess catalase activity, experiments were performed using Amplex Red-based methodology. As catalase removes H₂O₂ from the solution, less Amplex Red is converted into the highly fluorescent molecule resorufin. Fig. 3 shows a series of experiments performed in the presence or absence of nanoparticles. The addition of H₂O₂ to a reaction mixture in the absence of nanoparticles led to high levels of fluorescence, as anticipated. The addition of WO₃ nanoparticles, which have not been reported to possess catalase activity, were used as a negative control; these nanoparticles had no effect on the fluorescence signal. However, the addition of CeO₂ or CeTiO₂ nanoparticles to reaction mixtures did reduce H₂O₂ levels thereby decreasing fluorescence, which indicates a catalase-like enzymatic activity. As proteins can bind to nanoparticles and thereby alter their properties (Mahmoudi et al., 2011), we have tested the effect of cell culture medium on the catalytic activity of CeTiO₂ nanoparticles. Nanoparticles were incubated in culture medium for 48 hr at 37°C. The nanoparticles were then transferred to catalase assay medium. In paired experiments with untreated nanoparticles, no significant difference in catalase activity were observed (data not shown), which suggests that the incubation conditions have no catalytic effect on the nanoparticles.

Observation of Catalytic Nanoparticles in Vitro

After a 24 hr growth period on cover slips, cells were cultured with 17 ng/ml CeTiO₂ nanoparticles for an additional day. Cells were then washed with HBSS. To determine if CeTiO₂ nanoparticles became associated with cells, microscopy experiments were performed. Surface plasmon resonance enhanced darkfield microscopy was employed to detect nanoparticles. Fig. 4 shows a surface plasmon resonance enhanced darkfield micrograph of CeTiO₂ nanoparticle-treated cells. As this high magnification optical micrograph shows, nanoparticles can be found throughout the cytoplasm of the cell, occasionally in clusters. They are, however, not detected in the nucleoplasm of the cell. In contrast, untreated cells did not reveal the presence of intensely scattering nanoparticles (data not shown).

To localize CeTiO₂ nanoparticles to specific intracellular organelles, transmission electron microscopy was performed. Cells were incubated in the presence or absence of nanoparticles followed by processing for thin section transmission electron microscopy. Fig. 5 shows electron micrographs of cells processed without (panel A) and with (panel B) prior treatment with CeTiO₂ nanoparticles. Nanoparticle-treated cells exhibit electron dense staining due to nanoparticle accumulation with the cells. The dark nanoparticles are found within the vacuolar apparatus of the cell. The nanoparticles could not be found in other organelles such as the nuclei and mitochondria of cells. In other thin sections (data not shown), CeTiO₂ nanoparticles were found in association with the plasma membrane, suggesting that endocytosis might be a mechanism of cell entry. Hence, these nanoparticles are delivered to specific intracellular regions, and are not randomly dispersed within cells.

Hemolysis and cytotoxicity

We first examined the ability of CeTiO₂ nanoparticles to rupture erythrocyte plasma membranes using hemolysis assays. Human erythrocytes were treated with 1 µg/ml to 10 ng/ml nanoparticles for 3 hr at 37°C. Erythrocytes in buffer only were used as negative controls whereas erythrocytes treated with Triton-X 100 were used to measure 100% hemolysis. After exposure to CeTiO₂ nanoparticles, no hemolysis could be detected (Fig. 6). Hence, these catalytic nanoparticles do not appear to influence the integrity of erythrocyte membranes.

Although CeTiO₂ nanoparticles do not promote hemolysis, it is possible that they affect the integrity of nucleated cell membranes. To determine if nanoparticles cause the cytoplasm to leak from cells, they were labeled with the fluorescent marker Calcein-AM. Cells were labeled with Calcein-AM as we have previously described (Clark et al., 2010), then used in a marker-release assay. Control cells were treated with buffer alone. Cells were treated with 1 % Triton ×100 as a positive control for Calcein release (Fig. 7). After a 3 hr incubation with CeTiO₂ nanoparticles (10 ng/ml to 10 µg/ml), no cytotoxicity was observed (Fig. 7). This range of concentrations was chosen because it includes biologically-relevant activity levels (see below). Hence, these nanoparticles did not display hemolytic and nucleated cell cytolytic activity.

Apoptosis

Having assessed the physical, chemical and toxicological properties of CeTiO₂ nanoparticles, we next evaluated their ability to protect living cells from H₂O₂-mediated apoptosis. In these experiments caspase-3 activation was measured using the CaspGLOW™ red active caspase-3 staining kit, which is a cell membrane-permeable molecule consisting of a fluorogenic dye and a DEVD moiety specific for caspase-3. Samples were incubated with CaspGLOW™ red active caspase-3 substrate in the dark for 30 min, then washed with HBSS and observed using fluorescence microscopy. The fluorescence labeling of activated caspase-3 was quantified. After an overnight incubation with CeTiO₂ nanoparticles, cells were treated with H₂O₂ (200 µM) or buffer for 3 hr at 37°C. Cells treated with 200 µM H₂O₂ in the absence of nanoparticles demonstrate high levels of apoptosis (~50%) compared with the low levels of apoptosis (~7%) found for cells treated with buffer alone in the absence of nanoparticles (Fig. 8, first and second columns). CeTiO₂ nanoparticles, which exhibit catalase activity (Fig. 3), protect cells from H₂O₂-induced apoptosis (Fig. 8). However, in the absence of exogenous H₂O₂, nanoparticles had no influence on the level of apoptosis (Fig. 8, third column vs. first column). Similarly, CeTiO₂ nanoparticles protected cells from H₂O₂-induced changes in cell shape (data not shown). This was anticipated because caspase 3 also cleaves ROCK-1 kinase, which disrupts microfilaments within cells thereby leading to cell shape changes (Sebbagh et al., 2001; Ndozangue-Touriguine et al., 2008). Although H₂O₂ causes a dramatic increase in the percentage of caspase-3 positive cells, this can be significantly reduced by treatment with CeTiO₂ nanoparticles.

Discussion

Due to their unique physical and chemical properties, nanoparticles are assuming a greater role in heterogeneous catalysis. Certain nanoparticle catalysts, such as CeO₂, CeTiO₂, and Pt nanoparticles, exhibit the ability to destroy reactive oxygen species in aqueous environments (e.g., Chen et al., 2006; Clark et al., 2011; Colon et al., 2009). This catalytic activity has attracted interest in the potential clinical utility of these nanoparticles in managing ROS-mediated disorders. Included among these disorders are: Alzheimer's disease, diabetic retinopathy, age-related macular degeneration, glaucoma and amyotrophic lateral sclerosis (Osborn, 2010; Kawaji et al., 2011; Wu et al., 1997; Kowluru et al., 2006; Caldwell et al., 2005; Kanwar et al., 2007). In an effort to develop a new therapeutic nanoparticle to manage ROS-mediated disease, we studied CeTiO₂ nanoparticles.

In the present study we have found that the endogenous catalytic activity of CeTiO₂ nanoparticles was sufficient to largely deflect the activation of cell death pathways induced by H₂O₂, a crucial step in many disease processes. One advantage of CeTiO₂ nanoparticles is their spherical shape, as illustrated for Ce_{0.875}Ti_{0.125}O₂ (Fig. 1) and CeTiO₂ (Zhu et al., 2012) nanoparticles. As mentioned above, this shape offers improved biocompatibility. Another advantage of CeTiO₂ nanoparticles is that their superoxide dismutase activity is slightly less than that of CeO₂ nanoparticles (Zhu et al., 2012). This is due to the fact that the objective is to reduce, not eliminate, ROS. ROS participate in normal intercellular communication pathways within tissues. Moreover, harmful effects at the cellular level were not found in two assays of cell damage. Finally, the effectiveness of CeTiO₂ nanoparticles in diminishing apoptosis was demonstrated. Hence, these nanoparticles have catalytic and physiological advantages of other nanoparticles for biological use, but with reduced potential to harm cells.

The ability of CeTiO₂ nanoparticles to deflect H₂O₂-mediated apoptosis suggests that the nanoparticles have access to exogenous H₂O₂. Our electron microscopy studies have shown that CeTiO₂ nanoparticles are primarily found within the intracellular vacuolar system (Fig. 5). Previous studies have shown that H₂O₂ enters cells via diffusion (Ohno & Gallin, 1985). Indeed, the exogenous addition of H₂O₂ can be detected within cells using H₂O₂-sensitive probes (e.g., Petty & Francis, 1985; Cao et al., 1993). Hence, intracellular nanoparticles would have access to H₂O₂.

The present study demonstrates the concept that CeTiO₂ nanoparticles are capable of reducing H₂O₂-mediated apoptosis in tumor cells. However, as cells may differ in their interaction with nanoparticles, the nanoparticles' ability to influence physiological outputs may vary among cell types (Mahmoudi et al., 2012). Hence, although CeTiO₂ nanoparticles possess catalytic activity, the manifestations of that activity may vary depending upon the specific cellular environment.

Currently, nanoparticles are being used or tested as contrast agents in magnetic resonance imaging, drug delivery vehicles, and mediators of directed hyperthermia. However, catalytic nanoparticles are not being used in patient care, although they have been tested in several animal models of disease. The advantages described above for CeTiO₂ nanoparticles might

make them a more attractive candidate for clinical trials. This study suggests that CeTiO₂ nanoparticles will be useful in pre-clinical studies and, if successful, may lead to a new nanoparticle therapy. To develop a clinically relevant animal model, we are presently studying experimental approaches to deliver these nanoparticles to regions of oxidative damage and to target specific tissues.

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Fig. 1. A representative TEM image of Ce_{0.875}Ti_{0.125}O₂ nanoparticles. (Bar=100nm)

Fig. 2.

The size distribution of $\text{Ce}_{0.85}\text{Ti}_{0.15}\text{O}_2$ nanoparticles is shown.

Fig. 3.

Catalase activity is expressed by nanoparticles. The ordinate plots the amount of Amplex Red fluorescence for each of the experimental conditions shown. Catalase activity is evidenced by the reduction in the conversion of Amplex Red. The blank sample (H₂O₂ alone) demonstrated considerable fluorescence. This level of fluorescence was not influenced by the addition of WO₃ nanoparticles. However, CeO₂ and CeTiO₂ nanoparticles dramatically decreased the fluorescence intensity, thus demonstrating catalase activity in these samples. (P values: Ce_{0.875}Ti_{0.125}O₂ vs. H₂O₂= P<0.0001; Ce_{0.875}Ti_{0.125}O₂ vs. WO₃ P=0.004; Ce_{0.875}Ti_{0.125}O₂ vs. CeO₂, not significant) (n=3 to 7)

Fig. 4.

Imaging CeTiO₂ nanoparticles with living cells using surface plasmon enhanced darkfield microscopy. The nanoparticles are observed as bright dots during darkfield microscopy. Note that the nanoparticles can be found in the cytoplasm of the cell, but are excluded from the nucleus (N). (100× objective) (n=3)

Fig. 5.

Electron microscopic observations of CeTiO₂ nanoparticles in cells. Untreated cells (panel A) and Ce_{0.875}Ti_{0.125}O₂ nanoparticle-treated cells (panel B) are shown. The nanoparticles appear as small electron dense dots in panel B. Note that the nanoparticles are present in the vacuolar apparatus of cells. Their exclusion from the nucleus is consistent with Fig. 4. (Bar = 2 μm.)

Fig. 6.

CeTiO₂ nanoparticles do not promote hemolysis. Hemolysis was quantified spectrophotometrically by measuring the absorption of hemoglobin at 540 nm. Ce_{0.875}Ti_{0.125}O₂ nanoparticles were incubated with erythrocytes at the concentrations indicated for 3 hr. at 37°C. No significant hemolysis could be observed. (The error bars are smaller than the size of the lines.) (n=3)

Fig. 7.

CeTiO₂ nanoparticles do not promote cytolysis of nucleated cells. Cytolysis was quantified using fluorescence by measuring the release of calcein into the supernatant (Invitrogen) using excitation at 494 nm and emission at 517 nm. Ce_{0.875}Ti_{0.125}O₂ nanoparticles were incubated with cells at the concentrations indicated for 3 hr at 37°C. No significant cytolysis could be observed. (n=3)

Fig. 8.

Nanoparticles protect cells from apoptosis. After cells were pre-treated with or without Ce_{0.875}Ti_{0.125}O₂ nanoparticles, they were subsequently treated with buffer alone or 200 μ M H₂O₂ for 3 hr at 37°C to induce apoptosis. Caspase 3 activation was detected using the CaspGLOW™ red active caspase-3 staining kit. Controls in the absence of both nanoparticles and H₂O₂, treated with H₂O₂ alone, treated with nanoparticles alone, and treated with both nanoparticles and H₂O₂ are shown. Significant reductions in apoptosis are seen for the cells treated with nanoparticles in the presence of H₂O₂, but not in the absence of H₂O₂ (H₂O₂ vs. H₂O₂ + nanoparticles P=0.0004; blank vs. nanoparticles only not significant). (n=3)